DYNAMICS OF POST-TRANSLATIONAL MODIFICATIONS ON HUMAN HISTONE H4 THROUGH THE CELL CYCLE

BY

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B.S., United States Air Force Academy, 2005

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Chemistry
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2006

Urbana, Illinois

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
11 AUG 2006	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Dynamics Of Post-Translational Modifications On Human Histone H4 Through The Cell Cycle	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **University of Illinois at Urbana-Champaign**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) **AFIT/CIA**	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**67**	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

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ABSTRACT

Detection and characterization of histone PTMs have been conducted by antibodies, Bottom Up MS, and Top Down MS. As analytical techniques, however, they are not germane to quantitative biology unless they can provide consistently reproducible data that can be used to extrapolate trends and make correlations in addition to answering questions regarding modifications singly and in combination. Hydrophilic interaction liquid chromatography (HILIC) is a 2D-LC fractionation technique that produces an acetylation-methylation dependent elution profile of oxidized H4 thereby allowing for more in-depth interrogation of the protein forms present in solution. The substrates for Top Down Mass Spectrometry (TDMS) are whole proteins, facilitating analysis of post-translational modifications singly and in combination. Here we show that the combination of HILIC and TDMS enables reproducible quantitation of histone H4 PTMs as the cell cyles with the ability to observe biological phenomena such as progressive methylation and to detect rare modified protein forms.

ACKNOWLEDGEMENTS

It has been a whirlwind year and I cannot begin to express the full measure of my gratitude to all involved. My principle thanks go to Dr. Neil Kelleher who was crazy enough to go along with the idea of completing a research masters degree in roughly 7 months. He was the "Jonny on the Spot Advisor" that I needed to create the momentum for this project and keep it going. Of course, not much would have gotten done if it weren't for Jim Pesavento – my mentor, teacher, and go-to guy. Jim, because of you I now know the ins and outs of ECD, manuscript writing, and vegetarian food in CU. We may not agree on much outside of lab but I have been enriched personally and professionally by our relationship and sincerely hope that our paths cross again. I cannot thank the other members of the Kelleher Research Group enough for accepting me as one of their own and making coming into lab an enjoyable experience. To the first-year crew (you know who you are): thanks for making my civilian college experience one that I will remember fondly, with many smiles and laughs. Kristin, we've both come a long way since orientation day, thanks for being my TV source, fashion consultant, and truly great friend. I look forward to hearing of the success that I know awaits you.

Finally, I wish to thank the USAFA and UIUC Departments of Chemistry as well as the Air Force Institute of Technology for giving me the opportunity to obtain this graduate degree.

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aαS1	α-amino acetylation at serine 1
aK16	ε-amino acetylation at lysine 16
aK12	ε-amino acetylation at lysine 12
aK8	ε-amino acetylation at lysine 8
aK5	ε-amino acetylation at lysine 5
ECD	Electron Capture Dissociation
FIRR	Fragment-ion intensity ratio
FTMS	Fourier-Transform mass spectrometry
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HILIC	Hydrophilic interaction liquid chromatography
HMT	Histone methyltransferase
mK20	Methylation at lysine 20
mR3	Methylation at arginine 3
MS/MS	Tandem mass spectrometry
PIRR	Protein-ion intensity relative ratio
PTM	Post-translational modification
SWIFT	Stored Waveform Inverse Fourier-Transform
TDMS	Top Down Mass Spectrometry

CHAPTER 1: INTRODUCTION

1.1 – Chromatin and the Cell Cycle

In actively growing and dividing cells, there are several functionally distinct stages that make up the cell cycle. At its simplest level, the cell cycle is a process composed of four distinct phases: G_1, S, G_2, and M. These phases occur in sequence as the cell commits to DNA replication, replicates its DNA, prepares for mitosis, and undergoes cell division, respectively. The sequential nature of the cell cycle requires control mechanisms through which the cell is 1) permitted to continue in the cell cycle, 2) stopped or slowed to allow for corrective action or 3) marked for apoptosis [1].

Essential to the cell cycle is DNA replication. Over 2 m of genomic DNA is compacted into a ~10 μm nucleus through a myriad of DNA associated proteins to form what is known as chromatin [2]. The major repeating unit of chromatin is the nucleosome. The nucleosome is a complex of approximately 146 bp of DNA wrapped around a histone protein octamer [3]. Each histone octamer is composed of two each of histones H2A, H2B, H3 and H4. Through protein-protein interactions, H2A and H2B form dimers while two each of H3 and H4 form tetramers. Together, two H2A/H2B dimers and one H3/H4 tetramer form the core octamer around which DNA binds.

Within chromatin organization there are two categories: heterochromatin and euchromatin. In heterochromatin, DNA is retained in compact organization. Heterochromatic regions are transcriptionally inactive either constitutively or facultatively. Facultative heterochromatin is a temporary silent state in which DNA is compacted for transcriptional inactivation. Euchromatin is the less compact chromatin

domain containing far more transcriptionally active genes. Regions of chromatin can vary between euchromatin and heterochromatin depending on the stages of the cell cycle. Necessary activation of a silent gene results in the formation of a euchromatin domain. Nucleosomes mediate the formation of these chromatin domains and hence play a strategic role in the transformation of genes from silent to active and vice versa [4, 5].

Regulation of chromatin structure, either for gene activation or inactivation, is a fundamental step in transcriptional control for eukaryotes [6]. Because of different microscopy techniques, much is known about the condensation state of chromatin as the cell progresses through the cell cycle. Chromatin is most condensed during mitosis. However, at the beginning of G_1, within the chromatin mass, 100 – 130 nm decondensed chromonema fibers are visible. These fibers progressively uncoil and straighten as the cell progresses from G_1 to S phase. By early S phase, the chromatin mass exists primarily as 60 – 80 nm chromonema fibers of up to 2 – 3 microns in length. At the highest level of decondensation during S phase, 30 nm chromatin, extending for tens to hundreds of kilobases in length, is observed [7]. Following S phase, chromatin condenses back to its original state maintaining local and global chromatin structure of the parent and daughter chromatids [6].

The mechanism of chromatin reformation through nucleosome deposition was debated until the late 1980s, at which time Vaughn Jackson conducted a serious of rigorous density labeling experiments. Nucleosome deposition simply refers to the arrangement of parental (old) and new nucleosomes on newly synthesized DNA. Two theories of nucleosome deposition were proposed: conservative and random. The former required that parental nucleosomes did not release from the DNA during replication and

maintained their pre-replication conformation. The latter emphasized a lack of parental template with random deposition of new nucleosomes following DNA synthesis. Jackson's experiments revealed newly synthesized H3/H4 tetramers were complexed with two each of parental H2A and H2B. Likewise, a new H2A/H2B dimer was complexed with two each of parental H3 and H4 and one each of parental H2A and H2B [8]. This old-new histone deposition pattern was later clarified to be the result of random association between old and new H3/H4 tetramers and H2A/H2B dimers [9]. By cross-linking histone octamers with formaldehyde, Jackson also examined the stability of histone deposition through the cell cycle. He showed that H3/H4 tetramers and H2A/H2B dimers do not dissociate as the cell cycles and remain tightly associated with the DNA to which they were originally bound. Additionally, density labeling data suggested that nucleosomes are deposited randomly with parental and hybrid octamers being arranged adjacent to one another [10]. Nucleosome deposition and rearrangement may provide a window of opportunity through which chromatin structure, activating or silencing, may be disrupted. However, biology has shown that an epigenetic mechanism must exist whereby chromatin structure is maintained through many generations [6]. Histones, the building blocks of the nucleosome, are thought to be integral parts of this mechanism.

1.2 - Histone Post-Translational Modifications and the Histone Code

The N-terminal tails of the core histones trigger differing levels of histone-DNA and histone-protein interactions through changing post-translational modifications [11]. Histone-DNA interactions are affected by changes in electrostatic charge while histone-

protein interactions depend on the creation of combinatorial recognition platforms at specific loci [12]. Great efforts have been made to catalogue the types and sites of modifications and ascertain their biological contexts. Such work is focused toward unlocking the "histone code" [11]. The histone code is premised upon the idea that consistent sets of modifications exist that together orchestrate epigenetic events [13]. As such, proteomic study of histone proteins seeks to understand how post-translational modifications affect chromatin structure and influence epigenetic events.

Histones are subject to acetylation, methylation, ubiquitylation, phosphorylation, and poly(ADP-ribosylation) [14-17]. These post-translational modifications (PTMs) are enzymatically orchestrated. While all core histones, H2A, H2B, H3, and H4, can be substrates for enzymatic modification, a more detailed discussion of the enzymatic modifications of histone H4 serves as the basis for this research.

The first modification to histone H4 is an N-terminal acetylation of the α amino group on serine 1. This modification is co-translational and is present on all nuclear histone H4 (except reported herein) [18, 19]. It stabilizes the newly formed histone and aids in maintaining its integrity through further processing. The next step in processing histone H4 involves transport from the cytoplasm into the nucleus. Early work in electrophoretic separation and density labeling of newly synthesized histone H4 led to the hypothesis that both a phosphorylation and acetylation take place before histone H4 is transported into the nucleus. However, since the majority of nuclear histone H4 is found to be unmodified, dephosphorylation and deacetylation must then occur rapidly upon entry into the nucleus [20]. While great efforts were made in characterizing nuclear PTMs, less concern was given to these cytoplasmic PTMs until the mid-1990s when it

was unequivocally determined that K5 and K12 are the conserved sites of internal diacetylation of newly synthesized cytoplasmic human histone H4 [21]. The enzyme responsible for this acetylation event was identified as human HAT B, a ~100 kDa cytosolic protein with a 44 kDa catalytic subunit [22]. Evidence for cytostolic phosphorylation was not found [21].

Following transport into the nucleus, newly synthesized, diacetylated histone H4, in a tetrameric complex with two copies of new histone H3, is deposited selectively on replicating DNA. The deposition of newly synthesized H3/H4 tetramers is orchestrated by the chromatin assembly factor 1 (CAF-1). CAF-1 is comprised of three subunits: p150, p60, and p48 [23, 24]. The p150 and p60 subunits are found colocalized with sites of DNA synthesis, indicating a selectivity for newly replicated DNA [25]. The small subunit, p48, is not only related to CAF-1 but is also known to be a subunit of human histone deacetylase HD1, thereby leading to speculation that p48 may be involved in the initial deacetylation of human histone H4 at K5 and K12 following deposition [24, 26, 27]. CAF-1 complexed with a H3/H4 tetramer is referred to as the chromatin assembly complex (CAC) [24]. Following deposition of the H3/H4 tetramer, two H2A/H2B dimers from the available pool of free histones join to form the stable octameric complex around which the newly synthesized DNA wraps.

Following nucleosome formation, a number of post-translational modifications to histone H4 are possible, the two most common being methylation and internal acetylation. These modifications occur on several of the residues present in the disordered N-terminal tail of histone H4. Lysine 20 (K20) and arginine 3 (R3) can be methylated. This modification is dynamic and can be either mono-, di- or tri- for the former and mono- or

di- for the latter. Lysines 5, 8, 12, and 16 (K5, K8, K12, K16) can be acetylated [28]. As all histone H4 is co-translationally acetylated at S1, the form that is acetylated at only one of the internal residues is referred to here as monoacetylated with higher order references continuing up to tetraacetylated.

Methylation was one of the first histone PTMs discovered and is very stable. PR-SET7 is the enzyme responsible for monomethylation of human histone H4 at K20 [4, 29, 30]. Trimethylation of K20 is accomplished by Suv4 [31]. Currently, no histone methyltransferase (HMT) has been identified in conjunction with dimethyl K20. Such an enzyme would be expected to act either as a dimethylase on unmethylated protein molecules or as a monomethylase for those molecules previously methylated by PR-SET7. Dimethylation of R3 has only recently been detected and quantified [32]. PRMT1 is hypothesized to catalyze dimethylation at R3, though monomethylation is the predominant species observed experimentally [33].

The biological relevance and importance of histone H4 methylation have been vigorously investigated. Monomethylation of K20 (mK20) has been linked to a variety of different biological phenomena. X inactivation by the X*ist* ncRNA triggers enrichment of mK20 along the targeted chromosome in ES cells indicating a function in gene inactivation [34]. Conversely, more recent studies have shown that mK20 correlates with transcriptional activation of the β-globin gene and has significant colocalization with H3 3mK4, a known activating mark [5, 35]. Additionally, it has been suggested that mK20 is the first step in the di- and trimethylation of K20 (2mK20 and 3mK20, respectively) [36]. In support of a connection between mK20 and 2mK20, it has been demonstrated that in HCF-1 depleted cells there is a direct relationship between a

decrease in mK20 and increase in 2mK20, suggesting a specific switch between these two modifications [37]. Biologically, dimethylation of K20 is associated with heterochromatic regions on *Drosophila* polytene chromosomes [4]. Similarly, 3mK20 has been found to be localized to heterchomatic regions and correlates with H4 hypoacetylation giving more credence to the idea that 3mK20 functions as a silencing mark [38]. Methylation of R3 is suggested as an activating mark within the histone code. Following mR3 formation the enzyme p300 is recruited to selectively acetylate K8 and K12 [39]. Such acetylation is indicative of euchromatin. Additionally, PRMT1, the HMT responsible for mR3, is a nuclear receptor coactivator suggesting a role for mR3 in the transcription process [33].

Table 1.1. Summary of identified biological links for histone H4 methylation states.

Modification	Biological Link
1mK20	heterochromatin-X inactivation [34], activation of β-globin gene [35], colocalization with H3 3mK4 [5]
2mK20	heterochromatin-*Drosophila* polytene chromosomes [4]
3mK20	Hypoacetylation [38]
mR3	p300 recruitment, hyperacetylation [39]

Acetylation is a more transient modification, maintained through the coordinated actions of histone acetytransferases (HATs) and histone deacetylases (HDACs). Only lysine residues are acetylated generating a net decrease in positive charge. Of the possible sites of acetyation, it has been found that there exists a C- to N-terminal hierarchy wherein aK16 > aK12 > aK8 > aK5 [28, 40]. Acetylation at these sites can be initiated by a number of different HATs. p300 is a general acetyltransferase and can

acetylate any of the noted residues whereas other HATs are known to act selectively on a single residue [41]. Biologically, histone H4 acetylation is most closely linked with an open chromatin conformation that may facilitate access by transcription factors and chromatin remodeling complexes [42].

The notion of a histone code relies upon the understanding of PTMs singularly and in combination. Direct impacts on chromatin structure through changes in electrostatic charge and indirect impacts through recruitment of transcription factors and other proteins are all motivating factors to the study of histone PTMs. However, such study is non-trivial. If only methylation and acetylation are considered as possible PTMs on histone H4, there exist approximately 80 protein forms that could result in a single cell. Consequently, technology development has endeavored to ease the burden of complexity on histone PTM researchers.

1.3 – Two-dimensional Liquid Chromatography

Separating differentially modified protein forms is the focus of much chromatographic research. In histone research, reverse phase-high performance liquid chromatography (RP-HPLC) has been used extensively to separate different histone proteins as a function of polarity. However, RP-HPLC cannot effectively separate post-translationally modified forms of the same protein. For this, a higher resolution chromatographic technique is required.

Hydrophilic interaction liquid chromatography (HILIC) uses a high concentration of organic solvent in the mobile phase to emphasize hydrophilic interactions between the solute and the stationary phase thereby allowing for the separation of protein forms based

on slight differences in hydrophilicity. This technique was originally developed using a polysulfoethyl aspartamide stationary phase, a strong cation exchange material [43]. At low concentrations of acetonitrile (<50%), a mixed-mode effect was observed in the elution profile of different amino acids. This mixed-mode effect was due to competing effects between electrostatics and hydrophilicity. However, at >70% organic content in the mobile phase, hydrophilic effects eclipsed those of electrostatics and became the governing force in the elution profile [43].

The techniques presented by Alpert in 1990 were evaluated for utility in separating post-translationally acetylated histones in 1996 [44]. Whole histones separated from Friend erythroleukaemic cells were lyophilized and subjected to RP-HPLC to separate the core histones. The H4, H2A.1, and H2A.2 fractions were then subjected to HILIC on a SynChropak CM300 column. As was shown by Alpert, positively charged amino acids are highly hydrophilic. When a residue is acetylated there is a decrease in positive charge and hence a decrease in hydrophilicity causing elution of hyperacetylated histones and retention of hypoacetylated histones. The separation of different acetylation states by HILIC was followed by the separation of different methylation states in 2002 [45]. Unlike acetylation, histone methylation does not cause appreciable changes in the charge of individual amino acid residues. This makes electrophoretic separation of individual methylated protein forms a difficult part of histone PTM analysis. Application of HILIC, however, to histone fractions has allowed for the separation of histones based on methylation as well as acetylation state. Such separation allows for increased dynamic range and subsequently more in-depth analyses [32].

1.4 – Fourier-Transform Mass Spectrometry

Much work in histone PTM detection has used antibodies to track modified forms. However, studies have shown that antibodies raised against the same modification can report different trends [46]. Additionally, antibodies can optimally identify only one modification at a time, neglecting valuable information about the occurrence of modifications in combination. Nonspecific binding and cross reactivity also detract from the usefulness of antibodies for detection purposes. To overcome these problems and to extrapolate quantitative information about modification states and occupancy, a mass spectrometric approach has been developed to study histone PTMs.

Fourier-Transform Mass Spectrometry (FTMS) relies upon the entry of protein ions into an analyzer cell under the influence of a high magnet field. Gaseous protein ions are formed by electrospray ionization wherein samples are sprayed out of a nanospray robot at ~2 kV and then passed through a heated metal capillary where the protein ions are desolvated. These ions are then focused, resolved, and accumulated into coherent ion packets through a series of transfer octopoles and a quadrupole. Ion packets are passed into the cell of the mass spectrometer where they are trapped for excitation and detection [47]. Because of the magnetic and applied trapping electric fields, ions experience magnetron and cyclotron motion. During the excitation event, an applied radio frequency will cause ions with a resonant cyclotron frequency (a product of mass to charge ratio) to experience a larger cyclotron radius, spiraling away from the center of the cell. During this orbit, ions are attracted to two detection plates creating a measurable alternating current known as the image current. This measured image current can then be

used to determine the cyclotron frequency and hence the mass to charge ratio (*m/z)* of the excited protein ions [48, 49].

The two major mass spectrometric techniques used in histone PTM research are Bottom Up MS and Top Down MS (TDMS). Bottom Up MS uses enzymes to digest whole proteins, generating 1 – 3 kDa peptides, prior to MS analysis. This allows for peptide mass mapping or identification of PTM sites by MS/MS [28, 50]. This technique provides information on the presence or absence of individual PTMs on specific histones but the intial enzymatic digestion losses key information about the relative abundance of modifications as well as which modifications occur in combinations with one another, unless the modifications occur on the same peptide. In order to unambiguously determine the combination of PTMs on histone proteins, the intact protein must be processed and analyzed such that the identity and position of the PTM(s) are restricted to a single, known, preselected mass. The MS approach used in this study, Top Down MS, measures the intact mass of all protein forms in a sample, eliminating proteolysis and maintaining information regarding modifications in combination. When paired with tandem mass spectrometry (MS/MS) methods such as electron capture dissociation (ECD), data rich spectra allow for localization of PTMs to specific sites with 100% sequence coverage [51]. This thesis describes the combined use of HILIC and Top Down MS to characterize the PTMs present on unacetylated (Chapter 2) and monoacetylated (Chapter 3) histone H4 throughout the cell cycle.

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CHAPTER 2: METHYLATION DYNAMICS OF HUMAN HISTONE H4 THROUGH THE CELL CYCLE DETERMINED BY 2-DIMENSIONAL LC FRACTIONATION AND QUANTITATIVE TOP DOWN MASS SPECTROMETRY*

*Text taken from the following manuscript in preparation: Bullock, CR., Pesavento, JJ., Mizzen, CA., Kelleher, NL. Methylation Dynamics of Human Histone H4 Through the Cell Cycle Determined by 2-Dimensional LC Fractionation and Quantitative Top Down Mass Spectrometry. Pesavento grew HeLa cells and prepared H4 prior to 2D-LC and TDMS analysis. LeDuc assisted with statistical analyses.

2.1 – Introduction

The cell cycle is a linear sequence of events through which a cell commits to DNA replication (G_1), replicates its DNA (S), prepares for mitosis (G_2) and undergoes cell division (M) [2]. One of the key events of the cell cycle is DNA replication. Over 2 m of genomic DNA is compacted into a ~10 μm nucleus through histones and other DNA-associated proteins to form what is known as chromatin [3]. During S phase, while DNA is replicated, chromatin structure is strictly reformed behind the replication fork to maintain the local and global chromatin structure of the parent and daughter chromatids. Decondensation of chromatin during the passage of a replication fork through a loci may provide a transient window of opportunity through which silencing or activating chromatin structure may be disrupted. However, an epigenetic mechanism exists whereby chromatin structure – and therefore its activity – is maintained over many generations [4]. Histones and their complex pattern of post-translational modifications are thought to be integral parts of this mechanism.

The major repeating unit of chromatin is the nucleosome, a complex of approximately 146 bp of DNA wrapped around a histone octamer. The histone octamer is composed of two each of four core histone proteins: H2A, H2B, H3, and H4. Comprising ~25-30% of the mass of the individual histones, the N-terminal tails of these cores histones trigger differing levels of histone-DNA and histone-protein interactions through changing post-translational modifications [5]. These post-translational modifications affect electrostatic interactions as well as aid in the creation of combinatorial recognition platforms at specific loci [6]. Of the four core histones, human histone H4 and its modifications have been extensively characterized and studied. Possible modifications to histone H4 include: N-terminal acetylation (aαS1), serine 1 phosphorylation (pS1), arginine 3 mono- and dimethylation (mR3, 2mR3), lysine 5, 8, 12, and 16 acetylation (aK5, aK8, aK12, aK16) and lysine 20 mono-, di- and trimethylation (mK20, 2mK20, 3mK20).

Work done by our group and others has shown that acetylation and methylation vary as the cell cycles [7, 8]. These works, however, either provide a limited quantitative estimate or provide qualitative assessments of modification occurrence due to the limited ability of antibodies [8]. Here, using hydrophilic interaction liquid chromatography (HILIC) and a custom built 8.5 Tesla Q-FTMS, we report, reproducibly and quantitatively, the cell cycle dynamics of 5 unique histone H4 protein forms and identify and characterize two rare modified forms.

2.2 – Methods and Materials

Cell Culture. HeLa S3 cells (cervical adenocarcinoma) were grown in suspension in Joklik's modified MEM supplemented with 10% newborn calf serum and 100 U penicillin and streptomycin per mL.

Cell Synchrony. Growing cultures of HeLa S3 at a density of 2–3 x 10^5 cells/mL were synchronized by two 2 mM thymidine treatments as described previously [9]. The first thymidine block persisted for 16 hours, followed by a 9 hour release into media without thymidine which was followed by a second thymidine block that also persisted for 16 hours. Cells were then released into regular media. The degree of synchrony was assessed by flow cytometry of propidium iodide stained samples taken every hour following the second release. The representative mid-G_2 and mid-G_1 phases were determined to occur 8 and 14 hours post-release, respectively. Samples for biochemical analysis were collected by centrifugation at the desired intervals following release, washed twice with cold tris-buffered saline (TBS), flash frozen in liquid N_2 and stored at –80^{o}C prior to nucleus isolation.

Histone Preparation RP-HPLC purified H4 was prepared by chromatography of approx. 100 μg of crude HeLa S3 histone protein (with prior intentional performic acid oxidation) using a Vydac C18 column (4.6 mm i.d. x 250 mm) using a multi-step gradient from buffer A (0.1% TFA in 5% CH_3CN) to buffer B (0.094% TFA in 90% CH_3CN). H4 eluted as a single peak with minor H2A contamination and was recovered by vacuum drying in a Speed-Vac. Recovered fractions were dissolved in dH_2O, identity and purity assessed by SDS-PAGE and stored frozen at –20^{o}C prior to further analysis.

A total of three individual HeLa H4 HILIC experiments were performed and analyzed by FTMS for each of three time points: 0 h, 8 h, and 14 h. HILIC fractions of H4 were prepared by fractionating approx. 150 μg of RP-HPLC purified H4 as described previously [10] on a PolyCAT A column (4.6 mm x 150 mm; 3 μm, 1000 Å, PolyLC, Columbia, MD) with a multi-step gradient optimized for the resolution of modified H4 forms from buffer A (70% CH_3CN, 20 mM TEA, pH 4.0 with H_3PO_4) to buffer B (20% CH_3CN, 20 mM TEA, 500 mM $NaClO_4$, pH 4.0 with $H3PO_4$). Fractions of interest were partially dried in a Speed-Vac and H4 recovered by TCA precipitation, washed extensively with 20% TCA to remove residual salts, washed with acetone/0.1% HCl and then acetone, air dried and stored at –80^{o}C.

Mass Spectral Analysis by ESI-FTMS/MS. All data were acquired on a custom 8.5 Telsa Quadrupole-FT ICR MS with an ESI source operated in positive ion mode [11]. A quadrupole (ABB Extrel, Houston, TX) was used to select the 12+ or 14+ charge state of histone H4 species which were then accumulated in a rf only octopole equipped with a dc voltage gradient for improved ion extraction to the ICR cell [12]. The quadrupole window was set at ~40 *m/z* and centered around the H4 peak of interest. Typically, 10 μL samples were enough for more than 150 min. of stable nanospray using a NanoMate 100 (Advion) with low flow nanospray chips, providing ample time to acquire high-quality MS and MS/MS scans of one to four intact protein forms per sample. As the pre-MS purification was precipitation-based and not purified by RPLC, some samples contained varying amounts of chloride and phosphate. Both contaminants were partially removed by applying -25 V on the accumulation octopoles.

Electron Capture Dissociation. ECD was performed by applying 5 A through a dispensor cathode filament (Heatwave Technologies, Crescent Valley, BC). During the ECD event, ~10 V was applied on the grid potential while ~9 V were sent through the filament for optimal ECD. Typically, 15 cycles of ECD were performed, with individual irradiation times of 500 ms and a 10 ms relaxation time between cycles. All relative molecular weight (M_r) values and fragment masses are reported as neutral, monoisotopic species. ECD MS/MS spectra were internally calibrated using 3-4 identified $z^{\bullet}$ ions from the unmodified C-terminal region.

Ratios of Intact Protein Ions and Fragment Ions. For calculating the protein-ion intensity relative ratios (PIRRs) of histone H4, the intensity of top four most abundant isotopes ($^{13}C_5$, $^{13}C_6$, $^{13}C_7$, and $^{13}C_8$) were integrated to calculate intact abundance ratios for the 14+ and/or 12+ charge states. When possible, these were combined into a weighted average to report a final PIRR value for the sample, which was then used to calculate the relative amount of the corresponding molecular weight relative to the sum of the total. ECD generated 1+, 2+, and 3+ fragment ions, from which the $^{13}C_0$, $^{13}C_1$, and $^{13}C_2$ (when observed) isotope peaks were integrated and used to calculate the fragment-ion intensity relative ratios (FIRRs).

Software and Databases. A Web-based software and database suite, ProSight PTM (https://prosightptm.scs.uiuc.edu) [13, 14], was used to accelerate the characterization of histone H4 protein forms as previously reported [13]. An adapted version of THRASH [15] was used to convert raw data into monoisotopic mass values.

H4 PTM Quantitation. The HILIC chromatograph was integrated and the peak area of each fraction was calculated. For a single fraction, the area was then multiplied by the

relative ratio of protein ion intensities (PIRR) of each observed modified H4 form in the corresponding mass spectrum as previously described [16]. To calculate the percentage of each modified H4 form in the MS, the fragment-ion intensity relative ratio (FIRR) was used to determine the isomeric composition of a single intact molecular species [16]. In a few cases, a SWIFT-isolated window (<1.5 *m/z*) contained salt-adducted forms of a lower mass species. The amount of contamination was determined by fragment ions larger than c_{20}. The FIRRs were then corrected by subtracting out this contamination. Of the 93 HILIC fractions analyzed, one showed no measurable protein within the detection limit. The chromatographic area of this peak, representing < 1% of the total area and an estimated ~ 0.7% of the most abundant protein form (aαS1 + 2mK20), was subtracted out of the whole and removed from further calculations and analysis.

2.3 – Results and Discussion

HILIC Separation. As previously shown by our group, hydrophilic interaction liquid chromatography (HILIC) provides an acetylation and methylation dependent elution profile [17]. The most highly acetylated and methylated proteins elute earliest while the least modified protein forms elute latest. The addition of an acetyl group has the most dominant affect on separation as the acetyl-lysine no longer carries a positive charge. Nestled within each acetyl group are un- and multiply methylated molecules. As shown in Figure 2.1, three acetyl regions comprising 99.0% of histone H4 are observed. Of these three regions, the unacetylated protein forms encompass the largest percentage of the chromatographic area. On average, 76.6 ± 5.6% of the chromatographic area is due to unacetylated protein forms. As this unacetyl region contains the greatest amount of

protein and the least isomerically complex forms it was chosen for our initial mass spectral and statistical analyses.

Quantitation of Unacetyl, Multiply Methylated H4 Forms. HILIC fractions, collected every minute, were assigned an area relative to the total elution are of H4. This area was then multiplied by the MS protein-ion intensity relative ratios (PIRRs) so that any observed mass has a relative abundance value. If isomeric forms were present in an isolated intact mass the relative abundance value was then further broken down based on the fragment-ion intensity relative ratios (FIRRs). In this way, we have an estimated abundance for unique histone forms for one specific fraction. If the same forms were observed across multiple fractions, the HILIC area x PIRRs x FIRRs values were summed to report a final abundance value. By reiterating this method for quantitation, we have identified and quantified 7 forms of histone H4 (Table 2.1). These forms include 2mK20 (no N-terminal acetylation), aαS1 (no K or R methyl), aαS1 + mK20, aαS1 + 2mK20, aαS1 + 3mK20, aαS1 + mR3 + 2mK20, and aαS1 + 2mR3 + 2mK20.

Table 2.1. Identification and global quantitation of histone H4 PTMs from the shaded regions shown in Figure 2.1.

Modification	Monoisotopic Mass[a]	Δ*m*	% Abd. 0 h	% Abd. 8 h	% Abd. 14 h
2mK20	11289	28	0.28±0.11	nd[b]	nd
aαS1	11303	42	4±0.56	14±1.54	1±0.28
aαS1, mK20	11317	56	4±0.28	9±0.19	10±2.28
aαS1, 2mK20	11331	70	65±1.16	53±3.64	63±9.10
aαS1, 3mK20	11345	84	0.89±0.35	0.57±0.28	0.54±0.36
aαS1, mR3, 2mK20	11345	84	0.93±0.37	0.26±0.07	0.31±0.12
aαS1, 2mR3, 2mK20	11359	98	0.18±0.10	0.05±0.03	0.06±0.03

[a] Samples were intentionally oxidized at M84 prior to analysis [18]. Theoretical molecular weights given represent the oxidized forms, +32 Da from unoxidized.

[b] Not detectable. Either the form does not exist or exists below the detection limit of this experiment.

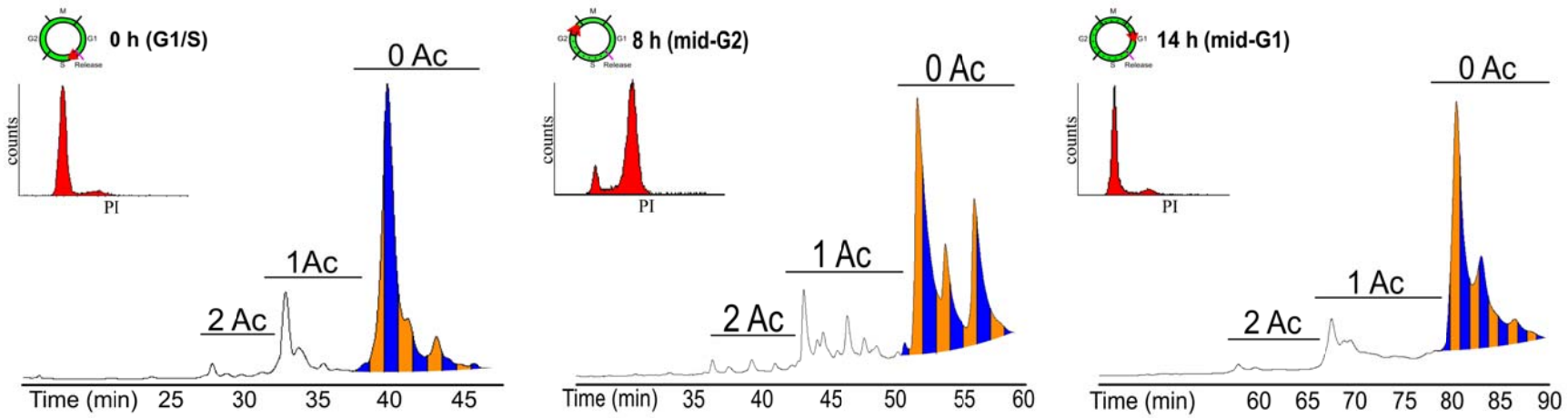

Figure 2.1. Representative HILIC chromatographs for each of the following cell cycle time points: 0 h, 8 h, and 14 h. These time points correspond to the G_1/S border, mid-G_2, and mid-G_1 of the cell cycle, respectively. As all histone H4 is co-translationally acetylated at aαS1 [1], a form that is acetylated at only the N-terminus and lacks any internal acetylations (K5, K8, K12, or K16) is referred to as unacetylated (0Ac). Labeled here are the diacetylated (2Ac), monoacetylated (1Ac), and unacetylated (1Ac) regions. The unacetylated region is shaded to delineate fraction collection boundaries every minute.

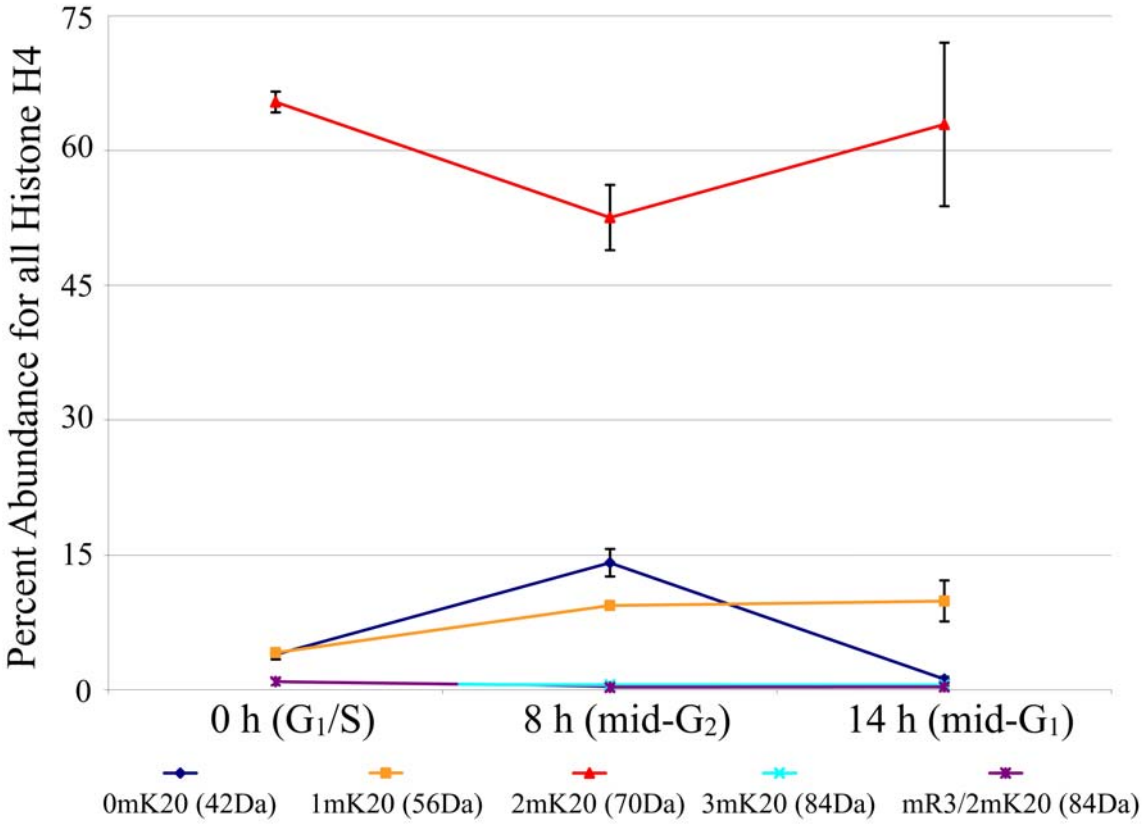

Figure 2.2. Percent abundance of the five major modification states identified in Table 2.1. Error bars represent the standard deviation from the mean as calculated from the three replicates for each time point.

Trends as the Cell Cycles. The cell cycle is a dynamic process dependent on variegated inputs from sometimes competing and synergistic factors. From the data presented herein, we show that changing patterns of histone H4 methylation are likewise dynamic. Histone synthesis and deposition is linked closely to DNA replication, a process that occurs during S phase [19]. As a cell doubles its nuclear content, there is a concomitant doubling of nucleosomes and hence histone proteins.

This addition of new histone H4 into the nucleus can be seen by the 3.5x increase of unacetylated, unmethylated H4 (aαS1) at 8 h (Figure 2.2). It has been shown previously that new histone H4 is transported into the nucleus as a diacetylated molecule (aK5 + aK12) but must be deacetylated to allow for chromatin maturation [20, 21]. As the cell cycles, this unacetyl form is progressively modified and at 14 h (mid-G_1) it exists as only 1% of the total histone H4 present in the nucleus. Additionally, there is a 2.25x increase in the amount of unacetylated, monomethylated H4 (aαS1 + mK20) at 8 h, representing mid-G_2. Rice, *et al.* showed that transcription of PR-SET7, the histone methyltransferase (HMT) responsible for monomethylating H4 K20, increases during G_2/M phase and declines during the transition to G_1 [22]. To date there has been no HMT identified to be responsible for dimethylating H4 K20, nonetheless, there appears to be a cell cycle dependent profile for this modification. Unacetylated, dimethylated H4 (aαS1 + 2mK20) decreases from 65% to 53% during the passage from the G_1/S border to mid-G_2. Between, mid-G_2 and mid-G_1 there is a return of aαS1 + 2mK20 to nascent levels. The modification profiles of aαS1 + 2mK20 and aαS1 are inversely correlated indicating that there could be a biological link between these two modification states. aαS1 + 3mK20 and aαS1 + mR3 + 2mK20 both represent less than 1% each of the total histone

H4 present in the nucleus. They show the highest levels at the G_1/S border with decreases into mid-G_2 (Table 2.1). Previous studies have shown aαS1 + 3mK20 to be linked to heterochromatin whereas aαS1 + mR3 + 2mK20 has been shown to be an activating mark [23, 24].

Rare modifications: 2mK20 and aαS1 + 2mR3 + 2mK20. Two rare protein forms were identified in this experiment: one with Δm = 28 Da and the other with Δm = 98 Da. The former, Δm = 28 Da, is due to dimethylation at K20 with no N-terminal acetylation (Figure 2.3).

We hypothesize that this protein form, representing 0.28% of total histone H4 at 0 h and not found at any other time point, is the result of a cellular mechanism to mark aαS1 + 2mK20 H4 for degradation. Early experiments done regarding N-terminal acetylation of histones showed that this modification acts as an initiating mark rather than existing simply as a co-translational or post-translational modification. It has been shown *in vitro* that an acetyl group is transferred to the serine of interest while the serine is charged on the tRNA, indicating that N-terminal acetylation of histones may occur prior to protein translation and elongation and therefore initiate histone synthesis [1]. As such, those proteins lacking this modification would have to experience removal following the initiation of protein synthesis.

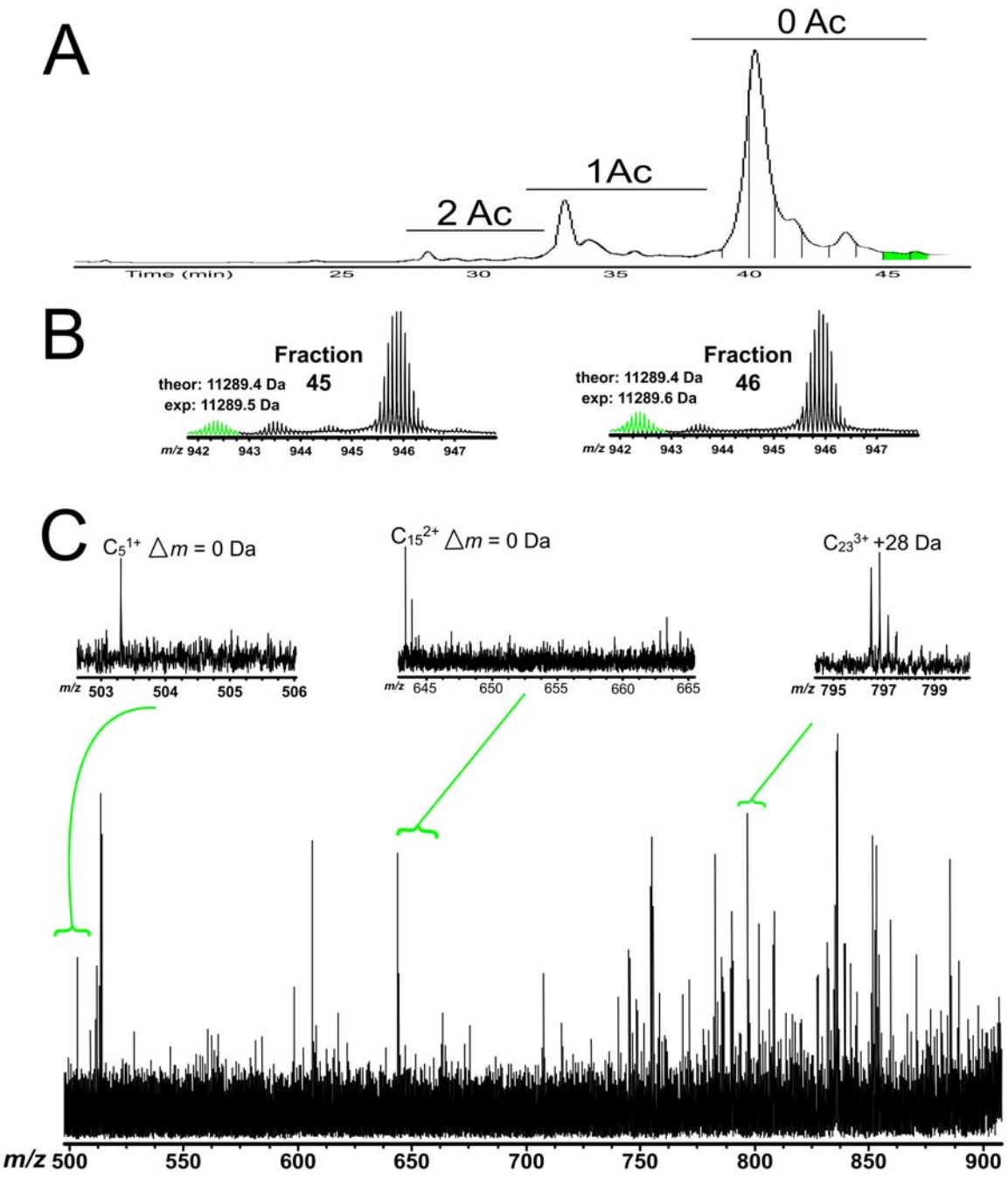

Figure 2.3. A) 0 h #1 HILIC chromatograph with fractions of interest shaded B) quadropole selected 12+ charge state for fractions 45 and 46. A monoisotopic mass of 11289 Da corresponds to a Δ*m* of 28 Da which is equivalent to 2 methylations. C) selected fragment ions from SWIFT/ECD of 11289.6 Da species in Fraction 46. All fragments before K20 show no modification whereas fragments after K20 exhibit a +28 Da shift. If an N-terminal acetylation was present, fragments before K20 would exhibit a +42 Da mass shift.

A second rare protein form, Δm = 98 Da, has only recently been reported [17]. This mass shift could be due to a number of isomeric forms including: aαS1 + aK5/8/12/16 + mK20, aαS1 + mR3 + 3mK20, and aαS1 + 2mR3 + 2mK20. While a +98 Da species was present at all time points, isomeric composition varied. At 8 h and 14 h, monoacetylation isomers (aαS1 + aK12/16 + mK20) were present in addition to the unacetylated form, aαS1 + 2mR3 + 2mK20. The 0 h time point consisted of a pure unacetylated form, determined by ECD to be aαS1 + 2mR3 + 2mK20 (Figure 2.4).

Protein arginine methyltransferase 1 (PRMT1) is hypothesized to catalyze dimethylation at R3, though monomethylation is the predominant species observed experimentally [25, 26]. The presence of aαS1 + mR3 + 2mK20 and aαS1 + 2mR3 + 2mK20 in the same cell population could be the first step in confirming dimethylase activity for PRMT1 and/or highlighting a mechanism besides that of deimination or demethylimination for the conversion of 2mR3 to mR3 [27, 28]. Interestingly, methylation of arginine 3, either mono- or di-, has only been shown to occur in conjunction with 2mK20. Such co-occurrence gives credence to the notion of a histone code wherein certain combinatorial sets of modifications jointly affect downstream events [5, 29]. This is not to say that methylation at R3 may not exist with other combinations of methylation at K20 at levels that are currently below our detection limit. For this experiment a detection limit of ~5 x 10^3 was calculated, equating to ~6500 out of 32 million nucleosomes in the human nucleus or roughly 0.02%.

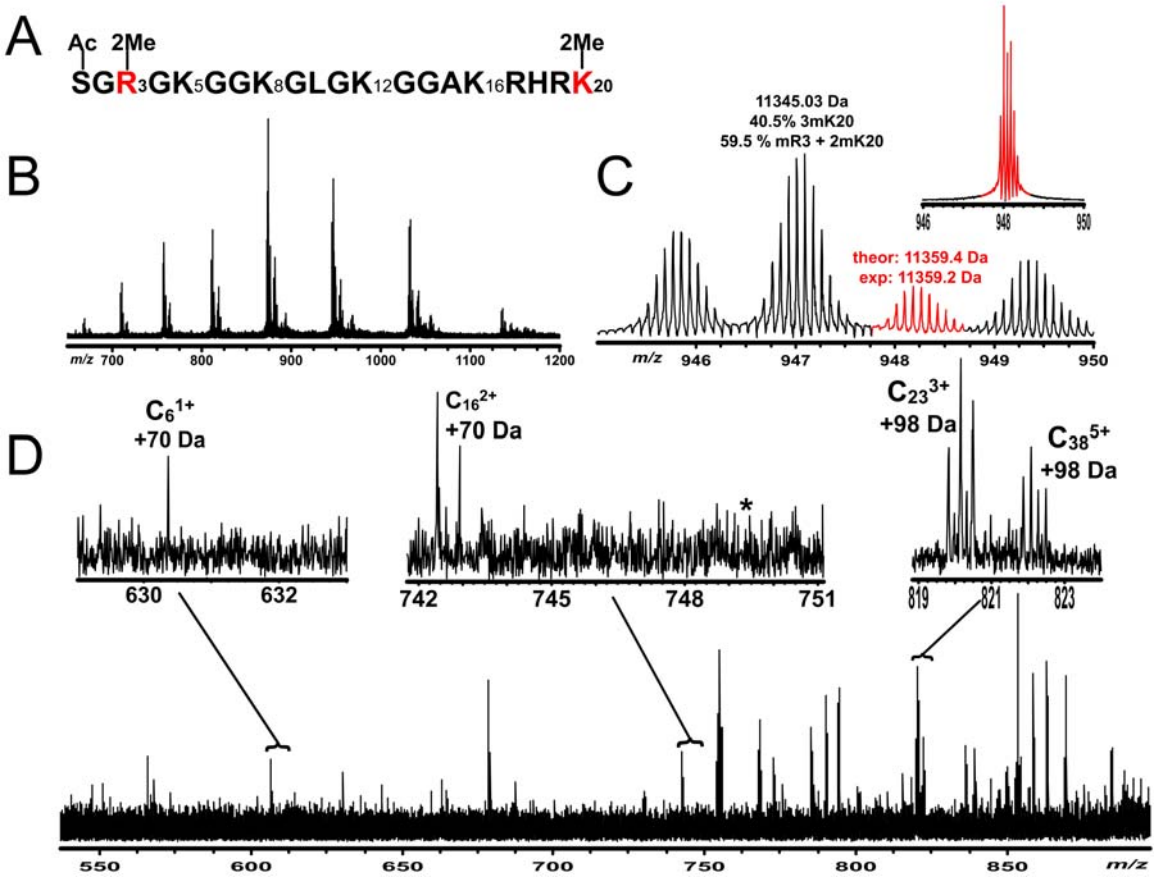

Figure 2.4. A) Amino acid sequence (1-20) of H4 N-terminus with identified PTMs based upon ECD fragmentation data. B) Broadband C) Quadropole selection of the 12+ charge state with subsequent SWIFT isolation of the 11359.2 Da form. D) ECD of 11359.2 form. +70 Da on fragment ions less than c_{20} correspond to an N-terminal acetylation (+42) and 2 methylations (+28). +98 Da represents the addition of 2 methyl group post-K20. The asterisk indicated where a c_{16} +84 would appear. Such an ion would indicate the presence of monoacetylation isomers.

Statistical Significance. In order to test the robustness of HILIC as a separation technique, all time point experiments were performed in triplicate. Fractions were collected every minute to allow for the utmost precision in PTM identification and quantitation. Following MS and MS/MS analysis, data was generated from each replicate for the 5 major protein forms listed in Table 2.1 ($\Delta m = 42 \rightarrow \Delta m = 84$). The data in Table 2.1 are the means measured across the three replicate experiments. In order to asses statistical significance, all data was entered into SAS Program and a square root transformation was performed (Figure 2.5). Following this transformation, ANOVA was conducted to test the differences between the square root of the percent abundance of the observed modification isoforms across the measured time points. The time by protein form effect (ie: the effect of the cell cycle on the presence of modified forms of histone H4) was found to be significant ($P < 0.0001$) so additional contrasts were run. A statistically significant difference ($P < 0.0001$, Bonferoni corrected) was found between 8 h and 14 h for unmethylated histone H4 (aαS1 + 0mK20). Such a difference can be biologically understood by the entrance of unmodified histone H4 into the nucleus during S phase (thereby being detected at mid-G2) and the progressive methylation of these histone molecules as the cell cycles until at 14 hrs when the 0mK20 form is markedly decreased.

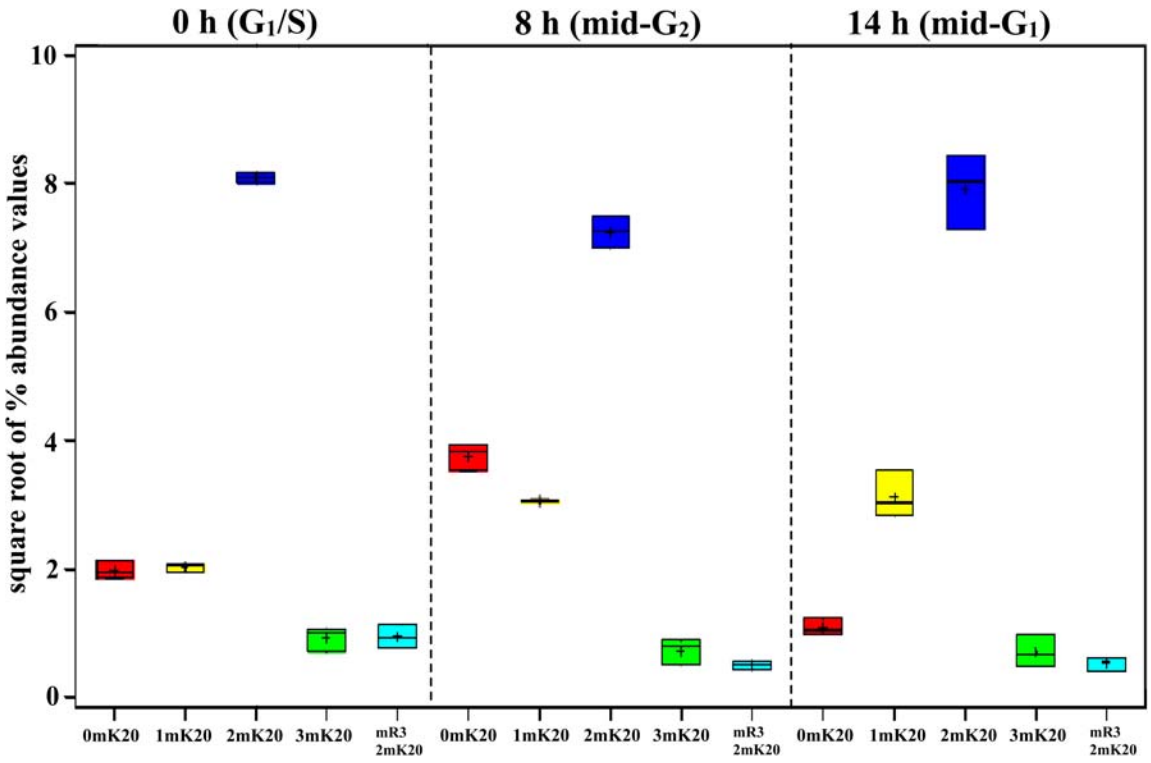

Figure 2.5. Statistical data generated by SAS following a square root transformation of the data presented in Table 2.1. Each colored box represents the variance of each measured value with the mean annotated with a solid line and the median with the plus sign.

2.4 – Conclusions

In conclusion, we have identified and quantified seven distinct protein forms of histone H4 that exist in the unacetylated region of the HILIC chromatogram, two of which are rare (2mK20 and aαS1 + 2mR3 + 2mK20). As the cell cycles, these forms demonstrate cell cycle dependent modification profiles that are reproducible across multiple experiments as shown by Figure 2.5. As precision is known to be relative to signal levels, it was found that for the most abundant protein form (aαS1 + 2mK20) coefficient of variation values within the square root transformation space were between 0.87% and 7.32%. This tightness of measurement validates the robust nature of HILIC for the separation of variably modified H4 forms.

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CHAPTER 3: TOP DOWN MS FOR CHARACTERIZATION OF CELL CYCLE CORRELATED CHANGES TO MONOACETYLATED HUMAN HISTONE H4 FOLLOWING ENRICHMENT BY HILIC

3.1 – Introduction

Acetylation was first identified as a post-translational modification in the 1960s [1]. Since that time, much work has been done to determine the effect of acetylation on the status of chromatin and the DNA contained therein. It is generally recognized that acetylation is associated with an open chromatin conformation and transcriptionally active regions of the genome [3]. Antibody studies have shown that euchromatic R bands contain intense levels of acetylation whereas constitutive and facultative heterochromatin had markedly less levels of acetylation [4]. Single gene studies on the β-globin gene have also confirmed the association of acetylated core histones with actively transcribed genes [5, 6].

Of the amino acids present in the N-terminal tails of the core histones, lysine is the substrate for acetylation. In histone H4, lysines 5, 8, 12, and 16 (K5, K8, K12, and K16) are acetylatable. The addition of an acetyl group decreases the positive charge on the modified histone. This decrease in positive charge weakens the interaction between the negative phosphate backbone of DNA and the positively charged N-terminal tail of the modified histone [3]. *In vitro* studies have shown that acetylation reduced the binding constant of the H4 N-terminus to DNA by six orders of magnitude [7]. The addition of the acetyl group also influences the positioning of nucleosomes relative to regulatory

DNA sequences and contributes to the creation of combinatorial binding platforms to which various transcription factors can bind [3].

Acetylation is the result of the coordinated actions of histone acetyltransferases (HATs) and histone deacetylases (HDACs). As such, the acetylation state of chromatin turns over constantly during interphase (G_1, S and G_2) [8]. Kinetic studies of the turnover rates for histone H4 acetylation and deacetylation have showed that there are two different populations of H4 molecules. The first has a turnover half life of approximately 7 minutes for monoacetylated H4 whereas the second population's turnover half life is between 200 and 300 minutes for monoacetylated H4. Similarly, the deacetylation half lives for these two populations are 3 – 7 minutes and 30 minutes, respectively [9]. Even under "slow" kinetic conditions, a number of turnovers are possible during the course of each phase of the cell cycle. To elucidate the cell cycle dynamics of acetylation, the work presented in Chapter 2 was extended to include monoacetylated histone H4.

3.2 – Methods and Materials

Cell Culture. HeLa S3 cells (cervical adenocarcinoma) were grown in suspension in Joklik's modified MEM supplemented with 10% newborn calf serum and 100 U penicillin and streptomycin per mL.

Cell Synchrony. Growing cultures of HeLa S3 at a density of 2–3 x 10^5 cells/mL were synchronized by two 2 mM thymidine treatments as described previously [10]. The first thymidine block persisted for 16 hours, followed by a 9 hour release into media without thymidine which was followed by a second thymidine block that also persisted for 16 hours. Cells were then released into regular media. The degree of synchrony was

assessed by flow cytometry of propidium iodide stained samples taken every hour following the second release. The representative mid-G_2 and mid-G_1 phases were determined to occur 8 and 14 hours post-release, respectively. Samples for biochemical analysis were collected by centrifugation at the desired intervals following release, washed twice with cold tris-buffered saline (TBS), flash frozen in liquid N_2 and stored at –80^{o}C prior to nucleus isolation.

Histone Preparation RP-HPLC purified H4 was prepared by chromatography of approx. 100 μg of crude HeLa S3 histone protein (with prior intentional performic acid oxidation) using a Vydac C18 column (4.6 mm i.d. x 250 mm) using a multi-step gradient from buffer A (0.1% TFA in 5% CH_3CN) to buffer B (0.094% TFA in 90% CH_3CN). H4 eluted as a single peak with minor H2A contamination and was recovered by vacuum drying in a Speed-Vac. Recovered fractions were dissolved in dH_2O, identity and purity assessed by SDS-PAGE and stored frozen at –20^{o}C prior to further analysis.

One individual HeLa H4 HILIC experiment was performed and analyzed by FTMS for each of three time points: 0 h, 8 h, and 14 h. HILIC fractions of H4 were prepared by fractionating approx. 150 μg of RP-HPLC purified H4 as described previously [11] on a PolyCAT A column (4.6 mm x 150 mm; 3 μm, 1000 Å, PolyLC, Columbia, MD) with a multi-step gradient optimized for the resolution of modified H4 forms from buffer A (70% CH_3CN, 20 mM TEA, pH 4.0 with H_3PO_4) to buffer B (20% CH_3CN, 20 mM TEA, 500 mM $NaClO_4$, pH 4.0 with H_3PO_4). Fractions of interest were partially dried in a Speed-Vac and H4 recovered by TCA precipitation, washed extensively with 20% TCA to remove residual salts, washed with acetone/0.1% HCl and then acetone, air dried and stored at –80^{o}C.

Mass Spectral Analysis by ESI-FTMS/MS. All data were acquired on a custom 8.5 Telsa Quadrupole-FT ICR MS with an ESI source operated in positive ion mode [12]. A quadrupole (ABB Extrel, Houston, TX) was used to select the 12+ or 14+ charge state of histone H4 species which were then accumulated in a rf only octopole equipped with a dc voltage gradient for improved ion extraction to the ICR cell [13]. The quadrupole window was set at ~40 *m/z* and centered around the H4 peak of interest. Typically, 10 μL samples were enough for more than 150 min. of stable nanospray using a NanoMate 100 (Advion) with low flow nanospray chips, providing ample time to acquire high-quality MS and MS/MS scans of one to four intact protein forms per sample. As the pre-MS purification was precipitation-based and not purified by RPLC, some samples contained varying amounts of chloride and phosphate. Both contaminants were partially removed by applying -25 V on the accumulation octupoles.

Electron Capture Dissociation. ECD was performed by applying 5 A through a dispensor cathode filament (Heatwave Technologies, Crescent Valley, BC). During the ECD event, ~10 V was applied on the grid potential while ~9 V were sent through the filament for optimal ECD. Typically, 15 cycles of ECD were performed, with individual irradiation times of 500 ms and a 10 ms relaxation time between cycles. All relative molecular weight (M_r) values and fragment masses are reported as neutral, monoisotopic species. ECD MS/MS spectra were internally calibrated using 3-4 identified $z^{\bullet}$ ions from the unmodified C-terminal region.

Ratios of Intact Protein Ions and Fragment Ions. Two calculations were made from the MS and MS/MS data. The first, protein-ion intensity relative ratios (PIRRs) is the result of the integrated intensities of the four most abundant isotopes ($^{13}C_5$, $^{13}C_6$, $^{13}C_7$, and $^{13}C_8$)

from the 14+ and/or 12+ charge states. Combined, these intensities report a final PIRR value which was subsequently used to calculate the relative amount of the corresponding intact molecular weight in the whole mixture. Fragment-ion intensity ratios (FIRRs) were calculated from MS/MS data generated by ECD. Isotope peaks ($^{13}C_0$, $^{13}C_1$, and $^{13}C_2$ – when observed) of 1+, 2+, and 3+ fragment ions were integrated and their intensities used to report a FIRR for each isomeric form present within an intact mass.

Software and Databases. A Web-based software and database suite, ProSight PTM (https://prosightptm.scs.uiuc.edu) [14, 15], was used to accelerate the characterization of histone H4 protein forms as previously reported [14]. An adapted version of THRASH [16] was used to convert raw data into monoisotopic mass values.

H4 PTM Quantitation. Using the entire elution area of H4, each HILIC fraction was assigned a relative chromatographic area. Each fraction area was then multiplied by the PIRR for each observed modified histone H4 form from the corresponding MS data as previously described [17]. FIRRs, generated by ECD, were used to calculate the isomeric composition of each intact molecular species [17]. Contamination by salt-adducted forms of a lower mass species occasionally occurred within the SWIFT-isolated window (<1.5 *m/z*) and were quantified by fragment ions larger than c_{20}. The FIRRs of interest were then corrected by subtracting out this contamination. Of the 37 HILIC fractions analyzed, one showed no measurable protein within the detection limit. The chromatographic area of this peak, representing < 1% of the total area was subtracted out of the whole and removed from further calculations and analysis.

3.3 – Results and Discussion

HILIC and TDMS Analysis. The coupling of hydrophilic interaction liquid chromatography (HILIC) with Top Down mass spectrometry (TDMS) enables increased dynamic range of $>10^4$ which aids in identification and quantitation of low abundant protein forms [18]. Whereas unacetylated histone H4 represents the most abundant protein form of nuclear histone H4, monoacetylated histone H4 is strikingly less abundant (Figure 3.1). This drop in abundance does not correspond to a drop in complexity. Indeed, twice as many monoacetyl isomers were identified as unacetyl isomers (Table 3.1). Acetylation can exist on lysine 5, 8, 12, or 16 (aK5, aK8, aK12, or aK16). In addition to acetylation, changing patterns of methylation are still observable at arginine 3 (mR3 and 2mR3) and lysine 20 (0mK20, 1mK20, 2mK20, and 3mK20). Of the 24 acetylation-methylation isomers possible, 14 were identified in this study. Previous work by our group identified 15 monoacetyl isomers of histone H4 in asynchronously grown cells with the additional isomeric form being aαS1 + 2mR3 + aK16 + 2mK20 [18]. The following 9 isomers have yet to be reported: aαS1 + aK5/8/12 + 3mK20, aαS1 + mR3 + aK5/8/12 + 2mK20 and aαS1 + 2mR3 + aK5/8/12 + 2mK20.

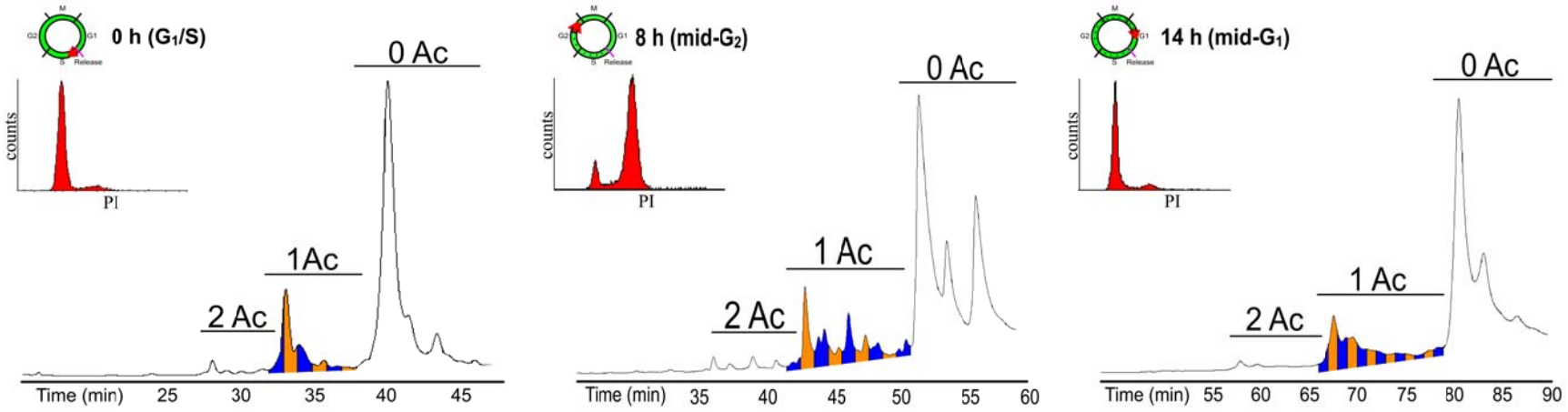

Figure 3.1. Representative HILIC chromatographs for each of the three cell cycle time points: 0 h, 8 h, and 14 h. As indicated by the FACS profiles, these times correspond to the G_1/S border, mid-G_2, and mid-G_1, respectively. Approximately 150 µg of histone H4 protein was fractionated for each HILIC using a multi-step gradient optimized for the resolution of modified H4 forms from buffer A (70% CH_3CN, 20 mM TEA, pH 4.0 with H_3PO_4) to buffer B (20% CH_3CN, 20 mM TEA, 500 mM $NaClO_4$, pH 4.0 with H_3PO_4). An acetylation dependent elution profile is visible with the diacetylated (2Ac), monoacetylated (1Ac), and unacetylated (0Ac) regions labeled for each chromatograph. Since all human histone H4 is known to have an N-terminal acetylation (aαS1) [2], di-, mono- and unacetylated refer to two, one or no acetylations at internal residues (K5, K8, K12, and K16). Alternately shaded regions reflect sample collection boundaries.

Table 3.1. Identification and global quantitation of histone H4 PTMs in the monoacetyl region of the HILIC chromatograph (Figure 3.1).

Modification	Monoisotopic Mass[a]	Δ*m*	% Abd. 0 h	8 h	14 h
aαS1, aK5	11345	84	nd[b]	0.45	0.02
aαS1, aK8	11345	84	nd	0.22	0.01
aαS1, aK12	11345	84	0.15	1.29	0.11
aαS1, aK16	11345	84	0.72	2.99	0.19
aαS1, aK5, mK20	11359	98	nd	nd	0.05
aαS1,aK8, mK20	11359	98	nd	0.21	0.07
aαS1,aK12, mK20	11359	98	0.12	0.67	0.51
aαS1,aK16, mK20	11359	98	0.43	1.95	1.37
aαS1, aK5, 2mK20	11373	112	nd	0.44	0.17
aαS1, aK8, 2mK20	11373	112	0.85	0.42	0.67
aαS1, aK12, 2mK20	11373	112	0.45	1.65	1.17
aαS1, aK16, 2mK20	11373	112	7.89	9.36	7.65
aαS1, aK16, 3mK20	11387	126	0.26	0.17	0.19
aαS1, mR3, aK16, 2mK20	11387	126	0.09	nd	0.14

[a] Samples were intentionally oxidized at M84 prior to analysis. Theoretical molecular weights given represent the oxidized forms, +32 Da from unoxidized.

[b] Not detectable. Either the form does not exist or exists below the detection limit of this experiment.

Patterns of Acetylation and Methylation as the Cell Cycles Nested within the data of Table 3.1 are the dynamics of both histone H4 monoacetylation and methylation as the cell cyles. Looking first at acetylation, a number of observations can be made (Figure 3.2, Figure 3.3). In accordance with previous reports, lysine 16 was found to be the predominant site of acetylation [19-21]. Additionally, the zip model whereby aK16 > aK12 > aK8 > aK5 can be discerned from the graphical data [20]. At 8 hours, the percent occupancy of lysine 5 and lysine 8 are approximately equivalent however 0 hour data shows no aK5 while at 14 hours aK5 is 2x less than aK8, thereby fully supporting the zip model (Figure 3.2). In all cases, aK12 > aK8 and aK16 >> aK12.

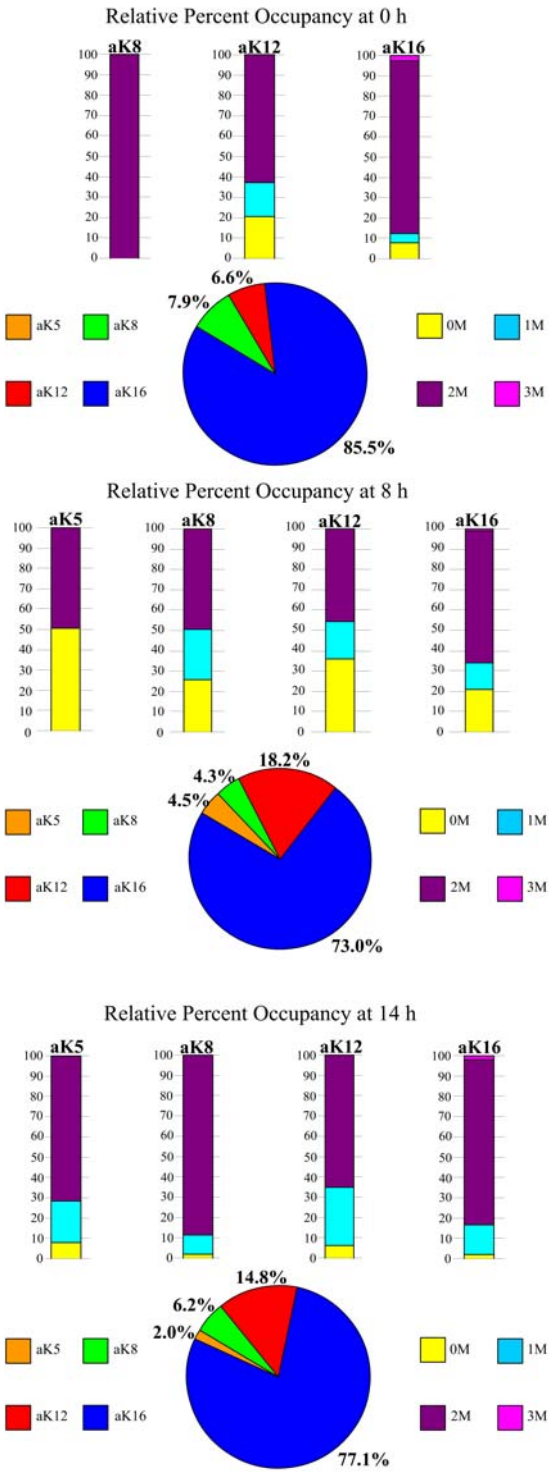

Figure 3.2. Pie charts delineate total percentages of each acetylation isomer (aK5, aK8, aK12, and aK16) calculated for every time point: 0 h – top, 8 h – middle, 14 h – bottom. Within each acetylation isomer the relative percentages of each of the four lysine 20 methylation isomers (0mK20, 1mK20, 2mK20 and 3mK20) are illustrated by sequential bar graphs.

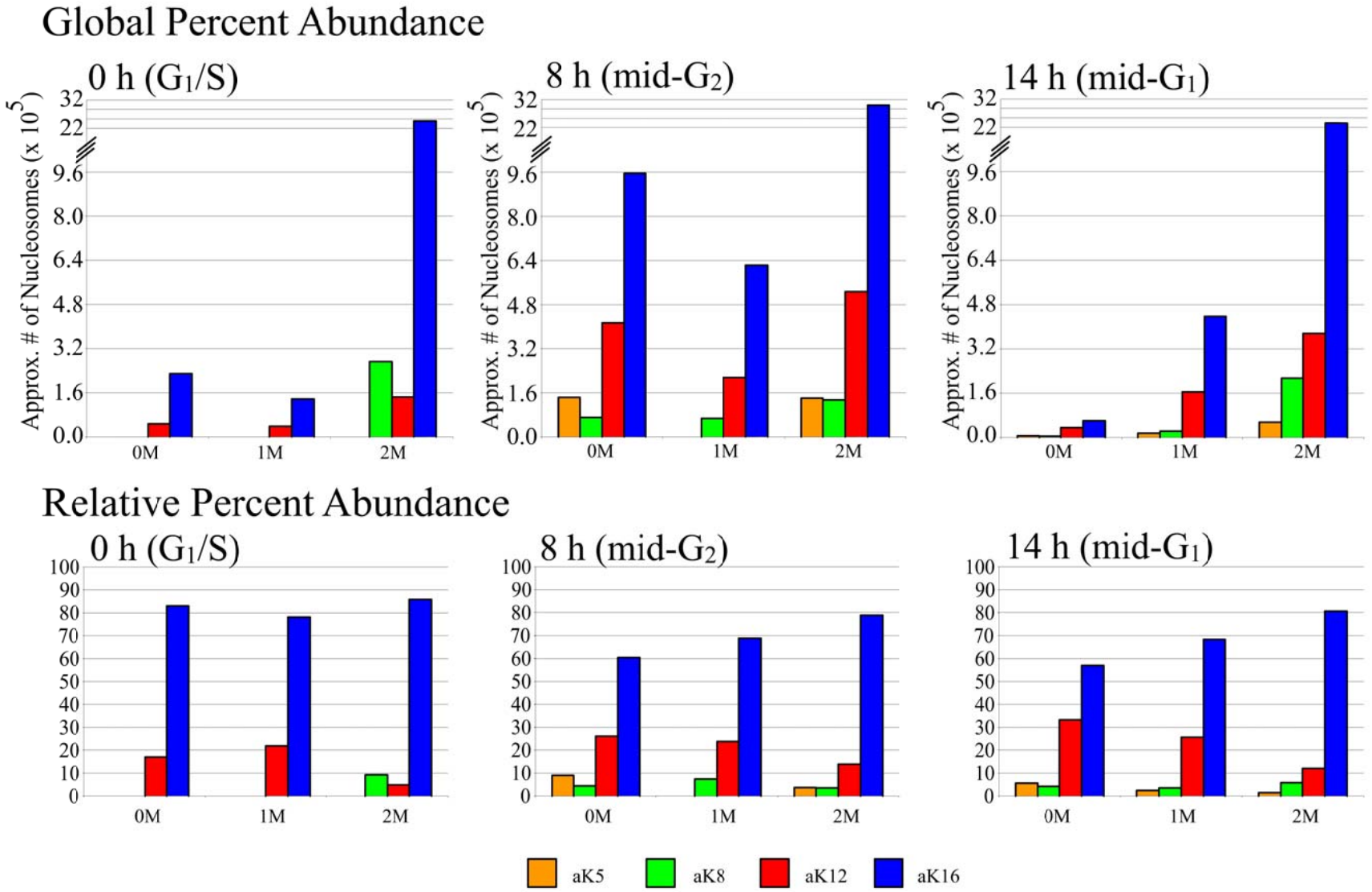
Global Percent Abundance
0 h (G1/S)
8 h (mid-G2)
14 h (mid-G1)
Approx. # of Nucleosomes (x 10^5)
32
22
9.6
8.0
6.4
4.8
3.2
1.6
0.0
0M
1M
2M
Relative Percent Abundance
100
90
80
70
60
50
40
30
20
10
0
aK5
aK8
aK12
aK16

Figure 3.3. cont. Graphical representation of global (top) and relative (bottom) percent abundance of each acetylation isomer clustered by methylation state at lysine 20 (0mK20, 1mK20, and 2mK20). Δm = 126 isomers were no included because 3mK20 and mR3 + 2mK20 are reported in conjunction with aK16 *only* hence providing no information about acetylation dynamics. Additionally, these two forms combined represent no greater than 0.17% of the total histone H4 present in the nucleus. Relative percent abundance was calculated such that all acetylation isomers present within a single methylation state sum to 100%. All values are corrected for chromatographic and mass spectral noise.

Across all acetylation states there is a noticeable increase in the level of acetylation observed at mid-G_2 (8 hours) with a subsequent decrease by mid-G_1 (14 hours) suggesting that acetylation is a mark associated with transcriptional activation of chromatin (Figure 3.3 – top).

Similar to unacetylated histone H4, monoacetylated histone H4 is subject to progressive methylation as the cell cycles. In fact, research suggests that progressive methylation is normally targeted to acetylated isoforms of histone H4 with 80 – 100% of all histone H4 being methylated by mitosis [22]. At 8 hours, ~75% of all monoacetylated histone H4 is methylated at least once. The global percentage of unmethylated, monoacetylated histone H4 molecules is highest at 8 hours before which time newly synthesized histone is imported into the nucleus. This new histone is then progressively methylated resulting in 0mK20 values < 0.5% for all acetylation sites at 14 hours (Figure 3.3 – top). Interestingly, the relative percentages of acetylation site abundance for 0mK20 does not change drastically from 8 hours to 14 hours implying that histone methylation by PR-SET7 occurs independent of acetylation site occupancy (Figure 3.3 – bottom).

3.4 – Conclusions

In conclusion, we demonstrate that acetylation occurs preferentially on K16 and that there is a global increase in the amount of monoacetylated histone H4 following S phase and continuing into G_2. Such an increase corresponds to an increase in transcriptional activity as the cell prepares for mitosis. Additionally, we show that

methylation occurs progressively on histone H4 molecules as the cell cycles with 2mK20 isomers being most abundant.

3.5 – References

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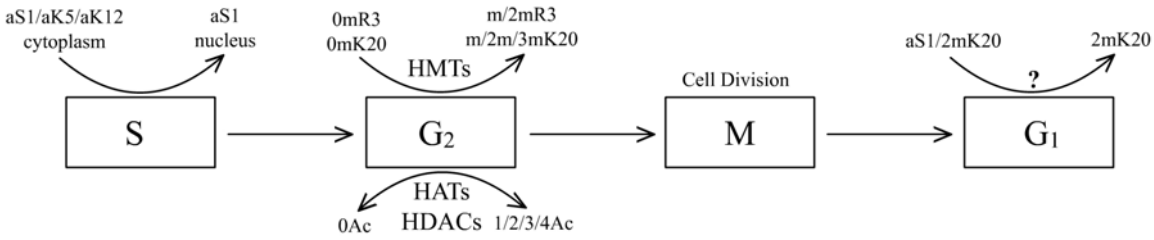
CHAPTER 4: CONCLUSIONS

Statements regarding histone post-translational modifications are more meaningful when made in the context of modifications singly and in combination across the whole protein. Since Top Down Mass Spectrometry (TDMS) interrogates the entire protein with no proteolytic digestion questions regarding modifications in combination can be answered definitively. Additionally, electron capture dissociation (ECD) is a tandem mass spectrometric technique that provides almost *de novo* sequencing of the N-terminal tail of human histone H4. In order to achieve the greatest clarity of isomeric identification during mass spectrometric analysis, hydrophilic interaction liquid chromatography (HILIC) was used to generate an acetylation and methylation dependent elution profile of oxidized human histone H4.

In this study we demonstrated the ability of these techniques to provide a wealth of information regarding various post-translationally modified forms of histone H4. In doing so, HILIC was shown to be a robust separation method that allows for experimental reproducibility. By analyzing the cell cycle time points in triplicate enough data was generated to create a statistical profile of H4 PTM identification and quantitation by HILIC separation and TDMS analysis. For the most abundant modification state (aαS1 + 2mK20) coefficient of variation (CV) values were 0.87%, 3.45%, and 7.32% at 0 h, 8 h, and 14 h, respectively. The precision represented by these CV values reflects the ability of HILIC to consistently separate oxidized human histone H4 into its post-translationally modified forms. In addition to reproducibility, the increased dynamic range of TDMS and HILIC made possible the identification and

quantitation of two rare protein forms (2mK20 and aαS1 + 2mR3 + 2mK20), both of which are biologically relevant.

Scheme 1



Two important biological phenomena, progressive methylation and acetylation dynamics, were also observed in this experiment (depicted in Scheme 1, above). Progressive methylation is the result of the actions of PR-SET7, an undetermined dimethylase, and Suv4 for lysine 20 and PRMT1 for arginine 3. Together, these enzymes modify newly imported H4 (aαS1) adding one, two, or three (lysine 20 only) methyl groups. This was shown to occur for both unacetylated and monoacetylated H4 in a cell cycle dependent manner. Internal acetylation state (0/1/2/3/4 Ac) and site (K5/8/12/16) are continually changing due to the activities of nuclear histone acetyltransferases (HATs) and histone deacetylases (HDACs). Human histone H4 exhibits an acetylation site hierarchy wherein aK16 > aK12 > aK8 > aK5. Experimental evidence detected the presence of 2mK20 (no N-terminal acetylation) implicating an, as of yet, unidentified role for HDACs in histone H4 regulatory control.

By identifying and characterizing the global post-translational modifications of human histone H4 as the cell cycles we have taken a small step toward greater understanding of the biological dynamics that exists within the human nucleus. Chromatin acts a determining force in the propagation and maintenance of genetic information. Yet, we have only begun to understand the many factors that affect

chromatin and its ability to function, globally and locally. The nucleosome is integral to the form and function of chromatin giving added importance to understanding the role of histone proteins in the bigger biological picture.

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